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Award Number: DAMD17-02-1-0422

TITLE: Breast Cancer Risk in Relation to Urinary Estrogen
Metabolites and Their Genetic Determinants: A Study
Within the Dutch "DOM" Cohort

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REPORT DATE: September 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE September 2004	3. REPORT TYPE AND DATES COVERED Annual (1 Sep 2003 - 31 Aug 2004)	
4. TITLE AND SUBTITLE Breast Cancer Risk in Relation to Urinary Estrogen Metabolites and Their Genetic Determinants: A Study Within the Dutch "DOM" Cohort			5. FUNDING NUMBERS DAMD17-02-1-0422	
6. AUTHOR(S) Rudolf J. Kaaks, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) International Agency for Research on Cancer 69372 Lyon Cedex 08, France E-Mail: kaaks@iarc.fr			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) Purpose and scope: We are conducting a large case-control study, nested within a prospective cohort, to estimate relative risk of breast cancer in post-menopausal women by levels of urinary estrogens and estrogen metabolites, and to examine the association between the concentrations of these hormones and breast cancer risk with polymorphic variations of a series of candidate genes, known to be implicated in the regulation of estrogen metabolites synthesis. Progress report: In this second year of the project, the personnel hired in Dr. Kurzer's laboratory (University of Minnesota) have been trained, and urinary analyses started. Unfortunately, due to a lack of sensitivity of the method used, and to a partial degradation of the estrogen metabolites in the DOM samples, the measurements were stopped after the analyses of 30 urine samples, since for more than half of the samples, less than 50% of the analytes could be measured. Tests to implement the detection limit of the method were done, with limited success. Conclusions: Our study encountered some major problems in the analyses of the urinary estrogen metabolites in the DOM samples, and therefore it is substantially delayed. Alternatives to the current method used for those analyses are proposed.				
14. SUBJECT TERMS Breast cancer, epidemiology, genetics, urinary estrogen metabolites				15. NUMBER OF PAGES 7
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	6
Reportable Outcomes.....	6
Conclusions.....	6
References.....	7
Appendices.....	7

INTRODUCTION

It has long been recognized that estrogenic steroid hormones, particularly 17 β -estradiol (E_2) can promote the development of breast tumors. Besides stimulating cell proliferation, there is increasing experimental evidence that estrogens may also be activated into genotoxic hydroxy metabolites that cause DNA mutations. In addition, some of the same metabolites may bind irreversibly to estrogen receptors, and thus stimulate cell proliferation permanently.

Major pathways through which hydroxy metabolites of estrogens (estrone [E_1] and estradiol [E_2]) are formed are the 16 α -hydroxylation pathway – which leads to formation of 16 α -hydroxy E_1 and estriol – and pathways that lead to 2- and 4-hydroxy (“catechol”) estrogens. Preliminary epidemiological evidence suggests that estrogen metabolism via the 16 α -hydroxy pathway is increased in breast cancer patients compared to controls, and an inverse relationship has been found between breast cancer risk and the ratio of urinary concentrations of 2-hydroxy/4-hydroxy or 2-methoxy/4-methoxy estrogens.

Amongst key enzymes involved in the natural conversion of estrogens to hydroxy estrogens are CYP1A1, CYP1B1, and CYP3A4. Furthermore, catechol-O-methyl transferase (COMT) is a key enzyme in the methoxylation of 2- and 4- hydroxyl groups, thus leading to methoxy estrogens. Methoxylation is a major pathway for the inactivation of the chemically very reactive catechol estrogens. In addition, experimental studies indicate that the methoxy metabolites inhibit tumor formation and development by decreasing cell growth, and inhibiting the formation of blood vessels in tumors.

Given these various observations, it has been hypothesised that breast cancer risk would be lower in women who produce more 2- and 4-methoxy estrogens relative to the levels of the corresponding hydroxyl estrogens. To examine the above hypotheses, we have started a case-control study nested within a large prospective cohort (the ‘DOM’ cohort, the Netherlands), with the following specific aims:

- 1) examine relationships of post-menopausal breast cancer risk with absolute and relative prediagnostic urine levels of 2-hydroxy, 4-hydroxy, 16 α -hydroxy, 2-methoxy and 4-methoxy metabolites of E_1 and E_2
- 2) examine relationship of polymorphic variants of genes encoding estrogen-metabolizing enzymes (CYP1A1, CYP1B1, CYP3A4 and COMT) to urinary levels of the various estrogen metabolites, as well as to breast cancer risk.

Our project is designed as a case-control study nested within a large prospective cohort, using urine and DNA samples collected from more than 50,000 women in the Dutch city of Utrecht and surroundings (“DOM” cohort). This cohort is unique, in that large volumes (50-100 ml) of urine were collected and stored for all study subjects. The majority of women in the cohort also provided a second (and even third) urine sample. The samples were stored in a large frozen warehouse. Relatively large volumes of urine (>10 ml) are needed to measure the estrogen metabolites, by gas chromatography coupled with mass spectrometry (GCMS). Cases and controls are selected among women who were post-menopausal at recruitment, and who did not use hormone replacement therapy. For about 60% of women who provided a

second urine sample within a time interval of about one year we also incorporated this second sample in our study, so as to improve exposure measurements.

BODY

For year 2, our work plan was (as in the "Statement of Work" of the original grant application):

- 1) Measurements of urinary measurements of estrogen metabolites by GC/MS (work subcontracted to the University of Minnesota): **Task 3 (months 13-24)**
- 2) Preparation of an exhaustive catalogue of polymorphisms in the *CYP1A1*, *CYP1B1*, *CYP3A4* and *COMT* genes by searching the literature, and by DHPLC analysis. Complete genotyping of all 300 breast cancer cases and controls: **Task 5 (months 13-18)**
- 3) Statistical analyses of relationships of genotypes with urinary estrogen metabolites and breast cancer risk: **Task 7 (months 12-24)**

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All tasks foreseen for year 2 had some delay:

Task 3: In this second year of the project, the personnel hired in Dr. Kurzer's laboratory were trained, and urinary analyses started in October 2003. However, after measuring about 30 samples, the analyses were stopped. In those first samples, only estrone and 2-methoxyestradiol were detected and measured in all subjects, while estriol and 2-hydroxyestrone were detected in 80% of the subjects, 2methoxyestrone and 2-hydroxyestradiol were detected in 50% of the subjects, 4hydroxy estrone, 16alfa hydroxyl estrone and 4hydroxy estradiol were measured in less than 30% of the subjects, and estradiol, 4methoxyestradiol and 4methoxyestrone were not detected at all in any of the subjects. We thought that these disappointing results may have been due to either the instability of some of the metabolites over time (the urine samples of the DOM cohort have been stored for more than 20 years at -20°C without any preservative agent), or to a problem of sensitivity of the method, or to both problems.

Since estradiol, estrone, 2-hydroxy estrogens and 16alfa hydroxy estrone had been successfully measured by immunoassays on urine samples from the same cohort (1) the degradation over time of the metabolites did not seem to be a major issue to us (even though some degradation may still have occurred, and absolute concentrations in the DOM samples may be lower than the concentrations found in fresh urines). We therefore checked if the current method used in Dr. Kurzer's laboratory was sensitive enough for the detection of estrogen metabolites in urine samples from post-menopausal women. Dr. Kurzer ran some analyses on forty post-menopausal urine samples, which had been stored with ascorbic acid at -20°C for one year. In those relatively "fresh" urine samples, the results obtained were generally comparable to those obtained when running analyses on the DOM samples, but for estradiol (that was measured on 48% of the subjects, compared to 0% in the DOM samples) and 2hydroxy estradiol (detected in 70% of the subjects, compared to the 30% in the DOM samples). For all other hormones,

problems of sensitivity were still observed. It thus appears that the major problem for the continuation of the study was the lack of sensitivity of the method used for the assays (the detection limit was finally determined to be about 2ng/ml).

To overcome this problem, some tests had been run in Dr. Kurzer's laboratory to increase the sensitivity of the method, by using more volume of the urine samples (30 ml rather than 15ml), and by concentrating the final solution injected into the gas chromatography/mass spectrometry. Unfortunately, none of these tests gave satisfactory results.

In the laboratory of the Nutrition and Hormones Group at the International Agency for Research on Cancer (IARC), a method for the analyses of estrogen metabolites in blood is being developed (supported by a small grant [R03] from the National Cancer Institute, Bethesda, USA; PI Dr Marlin Friesen). This method is also based on gas chromatography/mass spectrometry detection, but the use of a negative chemical ionization for the mass spectrometer detection increases the sensitivity of about 100 times compared to the sensitivity obtained by electron impact (the method of detection used in Dr. Kurzer's laboratory). We have good hopes that this method can be applied not only to measurements in blood, but also to urine samples. The setting up of the method at IARC started in 2002, and is planned to be completed by the end of 2004. Initial tests for sensitivity on standards and surcharged serum samples resulted in a detection limit of about 50pg/ml, well below the absolute concentrations found in urine samples for most of the metabolites. We propose to use this alternative method for the analyses of the urinary estrogen metabolites in the DOM cohort.

Task 5: A catalogue of polymorphisms has been prepared. We have not yet started genotyping, however, as we feel that our problems in measuring the urinary estrogen metabolites should first be resolved.

Task 7: Since no measurements of estrogen metabolites were available, statistical analyses could not be run.

KEY RESEARCH ACCOMPLISHMENTS

No key accomplishments have been realized in year 2.

REPORTABLE OUTCOMES

There are no reportable outcomes so far.

CONCLUSIONS

Our study encountered some major problems in the analyses of the urinary estrogen metabolites, and is therefore substantially delayed. Alternatives to the current method used for those analyses are proposed.

REFERENCES

- (1) **Rinaldi S**, Moret CN, Kaaks R, Biessy C, Kurzer MS, Dechaud H, Peeters PH, van Noord PA. Reproducibility over time of measurements of androgens, estrogens and hydroxyl estrogens in urine samples from post-menopausal women, *European Journal of Epidemiology*, 2003; 18:417-24.,

APPENDICES

None.